

## The effect of cassava (*Manihot esculenta*) leaf extract on COX-2 expression in the neutrophil cell culture exposed to the lipopolysaccharide of *Escherichia coli* (in-vitro study)

Zahara Meilawaty<sup>1\*</sup>, Agustin Wulan Suci Dharmayanti<sup>1</sup>, Dinar Prafitasari<sup>1</sup>

<sup>1</sup>Department of Biomedics, Faculty of Dentistry University of Jember, Indonesia

### ABSTRACT

**Introduction:** Neutrophils are cells which played an initial role in the inflammation area and can be induced by lipopolysaccharide (LPS). Activated neutrophils will secrete the COX-2 enzyme which is involved in arachidonic acid synthesis to produce inflammatory mediators. However, excessive inflammation can cause tissue destruction; thus, the body needs anti-inflammation agents. Cassava leaf contains biochemical component which has an anti-inflammation effect. This study was aimed to determine the expression of COX-2 in neutrophils administered with cassava leaf extract and the LPS of *E. coli*. **Methods:** This study was experimental laboratories (in vitro study) using human neutrophil cells culture. The samples of this study were divided into 4 groups, administered with 12.5% cassava leaf extract (T1); 25% cassava leaf extract (T2); control group which contained only neutrophils cells (C1); and control group contained neutrophils and LPS (C2). Human neutrophil cells culture was incubated in the 12.5% and 25% cassava leaf extract before exposed with the LPS of *E. coli*. COX-2 expression was observed with immunochemistry methods. **Results:** The COX-2 expression in the 12.5% cassava leaf extract group was significantly higher than the 25% cassava leaf extract group ( $p < 0.05$ ). **Conclusion:** Cassava leaf extract can inhibit the COX-2 expression and can be used as an alternative anti-inflammation agent.

**Keywords:** Anti-inflammation, cassava leaf extract, COX-2 expression, neutrophils.

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### INTRODUCTION

Inflammation is the body's physiological process in response to injury that involves arachidonic acid metabolism. This metabolic process will produce eicosanoids, which play a role in the processes of inflammation, angiogenesis, ovulation, implantation, and immunology. Eicosanoid

involvement in the process through three main enzymatic pathways, namely, the cyclooxygenase (COX) pathway, cytochrome P-450 cytochrome mono-oxygenase, but COX is a pathway that has been investigated as a common pathway involved in the inflammatory process.<sup>1</sup> COX enzyme is a catalytic enzyme for the formation of prostaglandins. This enzyme consists of two types,

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\*Corresponding author: Zahara Meilawaty, Department of Biomedics, Faculty of Dentistry University of Jember, Indonesia. Jalan Kalimantan, Krajan Timur, Jember Regency, East Java, Indonesia 68121. Phone: +62 812-3499-267; Email: [zahara.fkg@unej.ac.id](mailto:zahara.fkg@unej.ac.id)

namely COX-1 and COX-2. Most of the body's tissues produce COX-1 in physiological processes to control prostaglandin production and protect tissues and cells, such as in the stomach to protect the stomach tissue from stomach acid. While the COX-2 enzyme is an enzyme that is produced in a pathological state or by cells undergoing inflammation due to endotoxins, cytokines, growth factors and other injuries.<sup>2,3</sup>

Endotoxins are the most potent toxins produced by gram-negative bacteria, which will stimulate the immune system. Lipopolysaccharide (LPS) is one of the endotoxins found in bacterial cell walls and constantly released in the environment around bacteria. LPS exposure in tissue both in vitro and in vivo presented the presence of diseases caused by bacterial infections in humans, including periodontitis. LPS exposure will cause apoptosis, damage and inflammation in cells, tissues and organs. Several studies show that exposure to LPS *Escherichia coli* (*E. coli*) will damage endothelial cells so that LPS can penetrate the tissue, thus causing tissue damage. Likewise, LPS exposure to the gingival sulcus can cause periodontal tissue damage and trigger periodontitis.<sup>4-6</sup>

Neutrophils are the first and foremost immune cells undergoing migration and are in the inflammatory area to eliminate bacteria and their products, such as LPS. Microbial activity and chemicals will activate neutrophils by removing various inflammatory cytokines through the COX-2 pathway. However, excessive neutrophil activity will cause pathological conditions and tissue damage, including periodontal tissue damage.<sup>6-8</sup>

This neutrophil activity needs to be controlled with anti-inflammatory administration so that it can prevent tissue damage. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most effective anti-inflammatory drugs to suppress prostaglandin synthesis by inhibiting the production of the COX-2 enzyme. However, the drug has side effects, such as triggering stomach bleeding, prolonged bleeding time, and damage to kidney function. For this reason, it is necessary to explore alternative anti-inflammatory drugs that are safe and without causing side effects.<sup>1,3,9</sup>

Cassava (*Manihot esculenta*) leaves a misconception that plants are proposed as herbal medicines. In Nigeria, this leaf is used as a treatment for skin diseases, tumours,

conjunctivitis, abscesses, rheumatic fever, headaches, diarrhoea, and loss of appetite. Several studies have shown that cassava leaves show anti-haemorrhoid, anti-inflammatory and antimicrobial activities. This result indicated that cassava leaves contain several antioxidant compounds such as  $\beta$ -carotene (23-86 mg / 100 g), vitamin C (1.7 - 419 mg / 100g), vitamin A, anthocyanins (flavonoids), saponins, steroids, and glycosides. However, several studies mention the main component is flavonoids, and this is thought to have antioxidant and anti-inflammatory activity.<sup>2,9-12</sup> This study was aimed to determine the expression of COX-2 in neutrophils administered with cassava leaf extract and the LPS of *E. coli*. This study was necessary to determine the anti-inflammatory activity of cassava leaves which will be used as an alternative therapy for periodontal disease or periodontitis.

## METHODS

This research was an experimental laboratory with a post-test only control group design. This research was carried out in vitro using neutrophil cell cultures from human peripheral blood. Blood samples were taken from healthy donors, aged 30 years and agreed to the entire research process (informed consent).

Neutrophil cell culture was conducted by taking the blood sample for as much as 6 ccs from the brachial vein. The blood sample was then placed in two tubes containing heparin. After that, blood samples were centrifuged at 600 rpm at room temperature for 10 minutes. The results of centrifugation will produce two layers, namely blood cell layer and serum layer. Blood cell layers were separated, diluted with HBSS (the ratio of blood cells and HBSS was 1: 2) and homogenised. After that, diluted blood cells were placed in a falcon tube containing 3 ccs of hystopaque and 3 ccs of ficoll. Attention, blood cells must be placed slowly and carefully, to prevent damage to the ficoll layer because the gradient of blood cells is higher than ficoll.

The falcon tube was centrifuged at 1900 rpm at 200°C for 30 minutes. The results of centrifugation will produce 4 layers, namely erythrocyte, neutrophil, lymphocyte and plasma layers (from the bottom to the top). Then, the

plasma, lymphocyte, and ficoll layers were removed, while erythrocytes and neutrophils were resuspended with 6 ccs of HBSS. After that, the resuspension was drained into a tube contained 1 cc of 6% dextran and incubated for 60 minutes or until erythrocyte sedimentation occurs. This procedure was aimed to separate neutrophils and erythrocytes. Neutrophils located in the upper layer were then aspirated and transferred to tubes, added by 1 cc of HBSS and homogenised. Afterwards, the tube was centrifuged at 1700 rpm at 200°C for 10 minutes. After centrifugation, the top layer was removed, and 100 µl neutrophils were placed on well plates which were covered by a slipcover and contained the poly L-lysine. Then each well plates were added with 5 µL of fungizone, and 20 µL of penetrator, then incubated at the room temperature for 15 minutes. After incubation, the well plates were then washed using 1000 µL RPMI and viewed under an inverted microscope to determine contamination. If there was no contamination, the well plates were then added with 5 µL of fungizone and 20 µL of penetrator, then incubated at the room temperature for 30 minutes. 20 well plates were divided into 4 (four) groups, namely the treatment group (T1 = cassava leaf extract 12.5%; T2 = cassava leaf extract 25%) and the control group (C1 = neutrophil cells only; C2 = neutrophils and LPS).

Immunocytochemical treatment and analysis procedure was performed by putting 10 well plates of the treatment group plus 200 µl of 12.5% cassava leaf extract (5 well plates) and 200 µl of 25% cassava leaf extract (5 well plates). Then the well plates were incubated in the shaker incubator at the room temperature with 5% CO<sub>2</sub> for 15 minutes. After incubation, well plates were observed under an inverted microscope. The well plates were then resuspended with 1000 µl RPMI, homogenised and re-incubated at the room temperature with 5% CO<sub>2</sub> for 6 hours. After 6 hours, well plates were observed under a light microscope to determine the effect of cassava leaf extract on neutrophil cell culture.

Well plates in group T1, T2, and C2 were added with LPS *E. coli*. After that, the well plates were incubated at the room temperature with 5% CO<sub>2</sub> for 2 hours. Every hour, the well plates were observed under a light microscope to see the development of neutrophils. After 2 hours, the

well plates were washed with HBSS twice, fixed with absolute metabolism for 1 minute, washed with distilled water and incubated for 10 minutes. The distilled water was removed afterwards, the well plates washed with PBS and incubated for 10 minutes.

After the PBS was removed, the well plates were added with 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes and the PBS was washed 3 times for 5 minutes. Then, the well plates were added with 5 µl of primary antibody and incubated at 40°C overnight. After that, the well plates were washed, conjugated with streptavidin, incubated for 30 minutes, then washed again. The well plates were then added with a chromogen, incubated for 20 minutes, and washed. The washing procedure must used the PBS 3 times for 5 minutes.

The well plates were painted using hematoxylin-eosin (HE) and incubated for 50 seconds afterwards. The HE paint material was then removed and washed with distilled water for 10 minutes, then dried. Mounting and obstruction of COX-2 expression were performed under a light microscope with 400x magnification. The COX-2 expression was marked in dark brown.

## RESULTS

Neutrophils are one of the first immune cells to fight pathogenic agents, including bacteria and their products. Activated neutrophils will produce pro-inflammatory mediators as a form of host response to injury by activating the action of the COX-2 enzyme for the synthesis of arachidonic acid. The results showed that COX-2 expression in neutrophil cell cultures exposed by LPS (C2, mean  $\pm$  SD = 3.62  $\pm$  0.98) was lower than the treatment group (T1, mean  $\pm$  SD = 44.65  $\pm$  17.33; T2, average mean  $\pm$  SD = 28.95  $\pm$  9.89) (Figure 1).

Anti-inflammatory agents can suppress the activity of COX-2 enzymes, including anti-inflammatory agents derived from herbs. The activity of this therapeutic agent is directly proportional to the increase in therapeutic dose. This study showed that 25% cassava leaf extract (T2, mean  $\pm$  SD = 28.95  $\pm$  9.89) was having significantly higher antibacterial activity than 12.5% cassava leaf extract (T1, mean  $\pm$  SD = 44.65  $\pm$  17.33) , although the expression in this treatment group was higher than the control group (Figure 1).

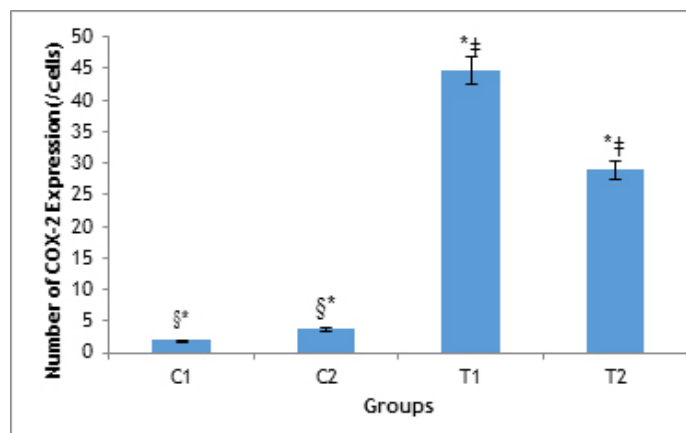


Figure 1. COX-2 expression in neutrophil cell culture

Notes:

The data presented are the average, standard deviation, and the results of multiple comparison tests

C1: control group 1 (neutrophil cell culture);

C2: control group 2 (neutrophil cell culture exposed to LPS);

T1: treatment group 1 (neutrophil cell culture exposed to LPS and administered with cassava leaf extract 12.5%);

T2: treatment group 1 (neutrophil cell culture exposed to LPS and administered with cassava leaf extract 25%);

\*: significant difference between control group (C) and treatment group (T) ( $p \leq 0.05$ );

‡: significant difference between treatment groups (T) ( $p \leq 0.05$ );

§ : no significant differences between control groups ( $p \geq 0.05$ )

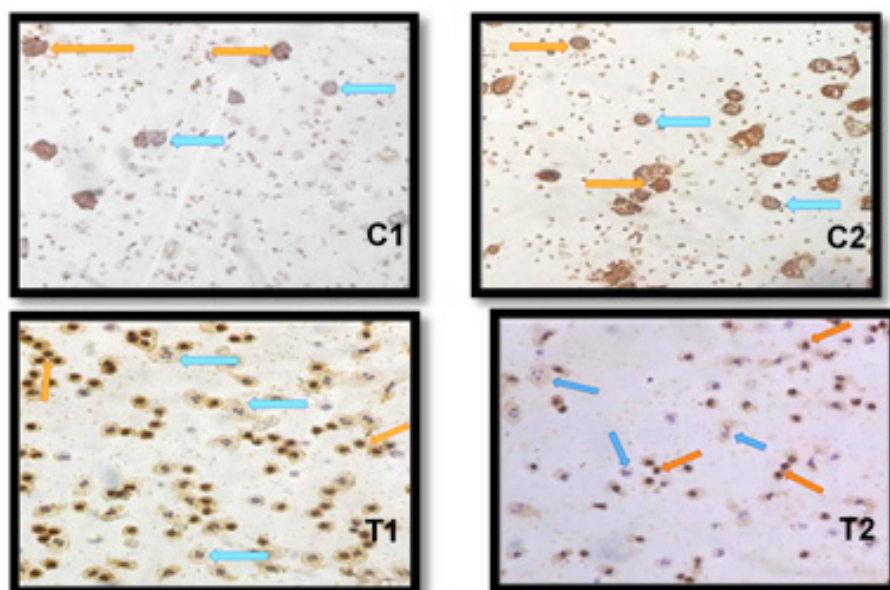


Figure 2. COX-2 expression in neutrophil cell culture (400x magnification)

Notes:

Blue arrows indicate no COX-2 expression;

Orange arrow shows the expression COX-2;

C1: control group 1 (neutrophil cell culture);

C2: control group 2 (neutrophil cell culture exposed to LPS);

T1: treatment group 1 (neutrophil cell culture exposed to LPS and administered with 12.5% cassava leaf extract);

T2: treatment group 1 (neutrophil cell culture exposed to LPS and administered with 25% cassava leaf extract)

Histological examination results showed that COX-2 was more expressed (dark brown) in the treatment group (groups T1 and T2) than in group C2. Also, neutrophils in the C2 group appeared to have lysis and few expressed COX-2, whereas the neutrophil cell membrane in the treatment group appeared to be more intact than the control group (Figure 2).

## DISCUSSION

Neutrophils are the main leukocytes in the peripheral blood circulation, approximately 40-70% of the leukocyte cells population. In the peripheral blood circulation system, neutrophils are silent or rested, but when exposed to injury (pathogenic agent), will be activated and produce inflammatory mediators such as leukotrienes and prostaglandins. This process will go through the synthesis of arachidonic acid through the cyclooxygenase enzyme, especially COX-2. LPS will trigger an increase in COX-2 enzyme activity which characterised by an increase in COX-2 levels expression in blood cells and tissues.<sup>1,3,7</sup>

The results of this study indicated that COX-2 expression in the control group (C2) was lower than in the treatment group ( $p \leq 0.05$ ), while some studies showed that expression of COX-2 levels have increased significantly with LPS exposure and triggered the inflammation process. The results in this study were probably caused by two causes, namely neutrophil lysis and the administration time administration of cassava leaf extract which was too short.

Lysis of neutrophils is probably related to the high LPS concentration in our current study so thus causing neutrophils to undergo lysis. This result was supported by the results of histological examination in which many neutrophils experienced lysis in the control group (C2). Lysis cells show that these cells die and do not functioned, as disruption of the COX-2 enzyme production for the synthesis of arachidonic acid. LPS concentration is likely to stimulate neutrophils to produce excessive reactive oxidative stress (ROS), thus causing cell death. ROS is a cell self-defence process against injury for phagocytosis and elimination of pathogenic agents, but excessive production of ROS will attack neutrophil cells themselves and cause cell death.<sup>7,8,11,13</sup>

Also, the time of administration of cassava leaf extract which was too short in the study is likely to be the cause of the anti-inflammatory effect of cassava leaf extracts is less optimum. This lack of optimum anti-inflammatory activity is likely correlated with low COX-2 expression in neutrophil cell cultures. Some studies state that anti-inflammatory agents, such as NSAIDs and herbal therapies, require an effective time of more than 4 hours, whereas this study uses an incubation time of 2 hours. Anti-inflammatory agents inhibit the activity and production of COX-2 enzymes after 4-6 hours of bacterial exposure. This condition is because neutrophils will start their activities some times after LPS invades cells, and the synthesis of arachidonic acid will begin 4 hours after bacterial exposure.<sup>8,9,14,15</sup>

Neutrophil culture cells in this study may experience twice the pressure, namely LPS and the anti-nutrient component (toxic component) of cassava leaf extract. LPS and cassava leaf extract are thought to be extracellular stimuli that cause disruption of COX-2 enzyme production and affect COX-2 expression in cells or tissues. There are two types of stimuli that affect the production of COX-2 enzymes, namely intracellular and extracellular stimuli. Intracellular stimulation consists of cytokines, growth factors, serums and the products of arachidonic acid synthesis products. Whereas extracellular stimulation can be in the form of biological injury (such as bacteria and bacterial products), physical, and chemical (toxic compounds).<sup>1,3</sup>

Cassava leaf extract used in this study is a crude extract that all active compounds, both toxic and non-toxic, are still contained therein. These compounds may cause disruption of neutrophil function in eliminating inflammation. Some studies mention that the extract of cassava leaves contains cyanogenic glucose compounds. This compound is the most toxic compound contained in cassava leaf extract. This compound can reduce the supply of energy in cells and cause injury to cells, although this compound functions as a protective tool for cassava plants from predatory pests.<sup>13,16-18</sup>

Also, the results of this study indicate that COX-2 expression in both treatment groups (T1 and T2) was significantly higher than in the control group (C2). The cassava leaf methanol extract



may be less potent as an anti-inflammatory because it contains only a few anti-inflammatory and antioxidant agents, so it is unable to protect neutrophils and reduce COX-2 activity. Anbuselvi and Balamurugan stated that methanol extract could reduce the content of flavonoids, phenols, vitamin C, steroids, terpenoids, and proteins. Though these compounds are important components for anti-inflammatory, analgesic, and antioxidant agents.<sup>16,19</sup>

Cassava leaf extract is still potential as an antioxidant and anti-inflammatory by maintaining the integrity of neutrophil cells. This property was supported by histological examination which showed neutrophil cell membrane in the treatment group was more intact than the control group and did not undergo lysis. Cassava leaf extract may have the ability to protect the surface of neutrophil cells so that LPS is not able to damage the neutrophil cell membrane, and no inflammatory reaction occurs. This condition is likely also cause low COX-2 expression in the treatment group. Several studies have shown the ability of cell protection by providing cassava leaf extract.<sup>12,15,20</sup>

The results also showed that there was a significant difference in COX-2 expression between treatment groups, where 25% cassava extract was more able to inhibit COX-2 expression in neutrophil culture cells than 12.5% cassava leaf extract. This shows that the dose of cassava leaf extract might influence the anti-inflammatory effectiveness of cassava leaf extract, the higher the concentration, the more potent anti-inflammatory activity.<sup>21,22</sup> Although the results of the study of Miladiyah et al. stated that the concentration of cassava leaf extract did not determine the magnitude of the activity of cassava leaf extract as an ingredient analgesics.<sup>17</sup>

Cassava leaf extract has the potential as an anti-inflammatory by inhibiting COX-2 expression in neutrophil cell cultures after being exposed by LPS from *E. coli*. Cassava leaf extract can be used as an alternative anti-inflammatory agent to reduce inflammatory reactions in periodontal disease, where the disease is caused by the bacteria *Porphyromonas gingivalis* which contains LPS as a potent virulence factor. Although this research still needs further research to find out the expression of COX-2 in neutrophil cell culture

with some observation time. Also, further research needs to be done about the bioactive components of cassava leaf extract as an anti-inflammatory ingredient.

## CONCLUSION

Cassava leaf extract can inhibit the COX-2 expression and can be used as an alternative anti-inflammation agent.

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